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Interactions between Double-Stranded RNA Regulators and the Protein Kinase DAI

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The interferon-induced protein kinase DAI, the double-stranded RNA (dsRNA)-activated inhibitor of translation, plays a key role in regulating protein synthesis in higher cells. Once activated, in a process that involves autophosphorylation, it phosphorylates the initiation factor eIF-2, leading to inhibition of polypeptide chain initiation. The activity of DAI is controlled by RNA regulators, including dsRNA activators and highly structured single-stranded RNAs which block activation by dsRNA. To elucidate the mechanism of activation, we studied the interaction of DAI with RNA duplexes of discrete sizes. Molecules shorter than 30 bp fail to bind stably and do not activate the enzyme, but at high concentrations they prevent activation by long dsRNA. Molecules longer than 30 bp bind and activate the enzyme, with an efficiency that increases with increasing chain length, reaching a maximum at about 85 bp. These dsRNAs fail to activate at high concentrations and also prevent activation by long dsRNA. Analysis of complexes between dsRNA and DAI suggests that at maximal packing the enzyme interacts with as little as a single helical turn of dsRNA (11 bp) but under conditions that allow activation the binding site protects about 80 bp of duplex. When the RNA-binding site is fully occupied with an RNA activator, the complex appears to undergo a conformational change.

Protein synthesis is modulated at several levels, most commonly at the stage of polypeptide chain initiation, and the phosphorylation of initiation factors plays a key role in controlling this process (reviewed in references 19 and 20). In mammalian cells, a regulatory mechanism involving an RNA-activated protein kinase and the eukaryotic initiation factor 2 (eIF-2) has been intensively studied. This initiation factor forms a ternary complex with GTP and Met-tRNA$_F$ and delivers the initiator tRNA to the ribosomal site of protein synthesis initiation. Discharged eIF-2 is subsequently released as a complex with GDP which must be replaced with GTP to permit the formation of another ternary complex in preparation for a further round of initiation. The factor is composed of three dissimilar subunits, α, β, and γ. Phosphorylation of a single residue, serine-51 of the α subunit, inhibits translation by trapping a second initiation factor, the guanosine nucleotide exchange factor (or eIF-2B), which is required to catalyze the substitution of GTP for GDP in the discharged eIF-2 complex. Phosphorylation of sufficient eIF-2 can sequester all of the guanosine nucleotide exchange factor, thereby preventing eIF-2 recycling and halting the initiation pathway.

In mammals, two protein kinases are capable of phosphorylating the α subunit of eIF-2 in this way (reviewed in references 20, 37, and 46). One of them, the heme-controlled repressor, is found chiefly in reticulocytes. It is activated by the absence of hemin, as well as by other stimuli, and serves to prevent the accumulation of globin in the absence of iron or heme. A second kinase, the double-stranded RNA-activated inhibitor (DAI; also referred to as P1 kinase, p68 kinase, P1/eIF-2α kinase, and PK$_{ds}$, etc.) is present in a wide range of tissues. DAI is an important element in the host antiviral response, and its synthesis is induced at the transcriptional level by interferon (reviewed in references 21, 54, 56, and 59). The enzyme is ribosome associated (11, 34) and normally exists in an inactive or latent state. Under some circumstances, DAI activation leads to the virtually complete abrogation of protein synthesis, while in other circumstances it may contribute to the selective translation of particular classes of mRNA (8, 24, 26, 36, 47, 60). It has also been implicated in cellular differentiation (23, 52), in the inhibition of cell proliferation (6, 51), in the heat shock response (10), and possibly in transcriptional induction (61, 64). Moreover, in yeast cells, the related protein kinase GCN2 mediates the growth response to amino acid starvation (9). As its name implies, DAI is activated by double-stranded RNA (dsRNA). Other polyanions such as heparin can also activate it, while small, highly structured RNA molecules such as adenovirus VA RNA suppress its activation (38). Thus, DAI is a pivotal cellular regulatory enzyme whose level and activity are modulated by factors of both viral and cellular origin.

The interactions between DAI and its RNA effectors are complicated and incompletely understood. The kinase is activated by dsRNA but not by DNA or DNA-RNA hybrids (22, 32, 35, 58). Single-stranded RNA, either synthetic or natural, is also inactive unless it can form extended hairpin-like structures (5, 22). There is no discernible sequence dependence for activation by dsRNA, and limited mismatching (44) and some modified bases (2, 45, 62) are tolerated, but the activity of dsRNA is reduced by ethidium bromide (1), suggesting that the topological form of the RNA duplex is important. Activation is accompanied by autophosphorylation of the kinase at multiple sites on serine and threonine residues (3, 11, 14, 30), and results in a change of substrate specificity such that the activated enzyme can phosphorylate the α subunit of eIF-2 and some other proteins (53, 58) but can no longer phosphorylate other molecules of DAI (29). Once activated, however, the phosphorylated enzyme is unaffected by the addition or removal of dsRNA (11, 58, 63).

Activation of DAI by dsRNA displays a paradoxical concentration dependence: the enzyme is activated by low concentrations of dsRNA (in the range of 10 to 100 ng/ml), but higher concentrations are decreasingly effective activators, giving rise to a bell-shaped activation curve (11, 22, 27,

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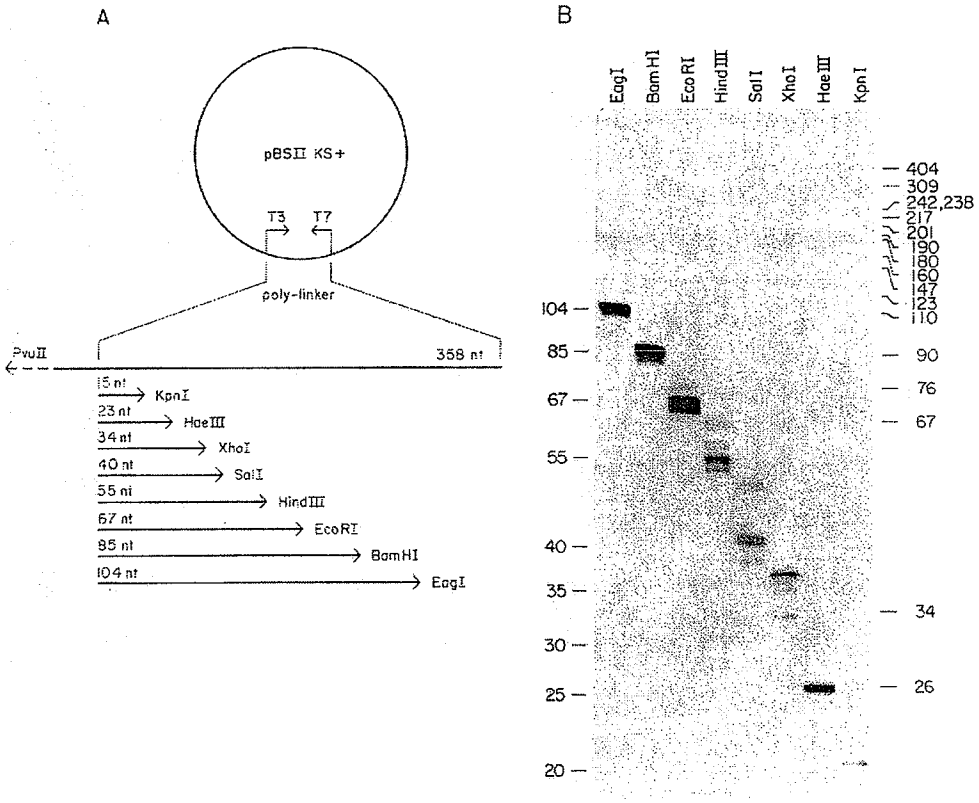

FIG. 1. Synthesis and characterization of RNA duplexes. (A) Schematic of short dsRNAs produced by transcription of pBSII KS+ polylinker sequences. The several transcripts of 15 to 104 nt synthesized by T3 RNA polymerase (rightward arrows) were annealed to the complementary 358-nt transcript (leftward arrows) synthesized by T7 RNA polymerase. After RNase digestion, the duplexes were purified by electrophoresis in nondenaturing gels. (B) Analysis of the purified dsRNAs in denaturing conditions. Samples of the radiolabeled dsRNAs were heated in formamide and resolved by electrophoresis in a 10% polyacrylamide–7 M urea gel. The fixed and dried gel was subjected to autoradiography. Size markers were single-stranded RNAs synthesized as described in part A (left) and pBR322-*Hpa*II DNA fragments (right).

33). High concentrations of dsRNA prevent the activation process but do not interfere with the activity of DAI once it has been activated by dsRNA at a lower concentration. The kinase also displays a stringent requirement for dsRNA chain length. Activation is reported to require a minimum of about 50 bp of duplex (18, 22, 32, 44), and there are indications that shorter duplexes may block activation at high concentration (44), as long dsRNA does. The ability of the enzyme to discriminate between dsRNA molecules on the basis of their chain length has implications for its regulation and the mechanism of DAI activation (37). Here we investigate the interactions of the enzyme with dsRNA molecules of specified sizes, studying binding and protection of dsRNA as well as activation and inhibition of the kinase. Our results define the dsRNA size dependence of the interaction and confirm that short duplexes which fail to bind stably and to activate the kinase can still interfere with activation mediated by longer duplexes. The data suggest that the minimal recognition element is a single helical turn but that there is an extended site for dsRNA binding which needs to be completely occupied for full enzyme activation.

MATERIALS AND METHODS

Synthesis of short dsRNAs. The plasmid pBSII KS+ (Stratagene, Inc., La Jolla, Calif.) was banded twice in CsCl, passed over a Bio-Gel A 15-m column, and then digested with one of eight enzymes (*Kpn*I, *Hae*III, *Xho*I, *Sal*I, *Hin*dIII, *Eco*RI, *Bam*HI, or *Eag*I), which cut in the polylinker, or with *Pvu*II, which cuts outside the region containing the polylinker and the T3 and T7 promoters (Fig. 1A). The DNA was incubated with RNase A to remove the last traces of RNA, treated with proteinase K, and extracted with phenol and chloroform. After ethanol precipitation, the DNA was added to transcription reactions containing T7 RNA polymerase (17) for the PvuII-digested template or T3 RNA polymerase (Stratagene, Inc.) for the other templates. Reaction conditions were as described previously (43), except that the concentration of GTP or CTP was reduced to 12 μM for labeling. The corresponding [α-^{32}P]ribonucleotide (from ICN Biomedicals Inc., Costa Mesa, Calif.) was present at a concentration of 500 μCi/ml. Single-stranded RNA was recovered after DNaseI digestion and phenol and chloroform extraction by ethanol precipitation. Each of the

T3 products (15 to 104 nucleotides [nt]) was mixed with an approximately equivalent amount of the complementary T7 product (354 nt), heated to 100°C, and annealed as previously described (29). Following digestion with both RNase T_1 and RNase A, dsRNA was isolated by treatment with proteinase K and deproteinization and then fractionated by electrophoresis in a nondenaturing 10% polyacrylamide–0.5× Tris-borate-EDTA (TBE) gel. The bands were detected autoradiographically, and each dsRNA was eluted into 10 mM Tris–1 mM EDTA –10 mM NaCl–0.5% sodium dodecyl sulfate (SDS), deproteinized, and ethanol precipitated. The dsRNA was dissolved in the same buffer without SDS, and its concentration was calculated from the specific activity.

Other RNAs. Longer dsRNAs (354 bp) were synthesized by transcription of the pGEM.GC plasmid (42). Reovirus dsRNA was provided by A. J. Shatkin (Rutgers University, New Brunswick, N.J.), and *Penicillium chrysogenum* and bacteriophage f2 sus3 dsRNAs were provided by H. D. Robertson (Cornell University Medical School, Ithaca, N.Y.). Labeled single-stranded RNA was purified from the T7 and T3 polymerase transcription reactions described above by electrophoresis through a 10% polyacrylamide–7 M urea–0.5× TBE gel.

Kinase assays. Reactions (10 μl) containing 2.5 μCi of [γ-^{32}P]ATP (ICN Biomedicals, Inc.) and 0.5 μl of DAI (about 5 ng) purified to the Mono S stage (29) were conducted essentially as described by Mellits et al. (42). The enzyme was added last to the other reaction components assembled on ice. Phosphorylation was visualized by SDS-polyacrylamide gel electrophoresis and autoradiography for 4 to 16 h by using an intensifier screen.

Nitrocellulose filter-binding assay. The nitrocellulose filter-binding assay was conducted by using a modification of the published procedure of Kostura and Mathews (29). Briefly, labeled dsRNA was incubated for 20 min on ice with the Mono S fraction of DAI under kinase reaction conditions, with bovine serum albumin (BSA) and calf liver tRNA both added to a concentration of 0.1 mg/ml but with labeled ATP omitted. After dilution with 10 volumes of wash buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) K^+ [pH 7.4], 0.1 mM EDTA), the reaction mixtures were immediately filtered in a slot-blot apparatus through a 0.45-μm-pore-size nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) that had been soaked for 1 h at room temperature in wash buffer containing 0.1 mg each of BSA and salmon sperm DNA per ml. Each well was washed with 200 μl of ice-cold wash buffer, and the filter was dried and exposed to autoradiography. Quantitation was done by scintillation counting of individual bands or direct scanning of the membrane with the AMBIS Imaging System.

Binding of dsRNA to Sepharose-bound DAI. A mixture of dsRNAs was partially degraded by incubation with RNase T_1 and RNaseIII (provided by H. D. Robertson) and then incubated with DAI immobilized on monoclonal antibody-Sepharose beads (13, 31) (from A. Hovanessian, Institut Pasteur, Paris, France) as described previously (40). The beads were sedimented and washed five times by resuspension and sedimentation. Of the input radioactivity, approximately 15% was recovered with the beads, 80% was recovered in the initial supernatant plus first wash fraction and 5% was recovered in subsequent washes. RNA was extracted from the beads and from the initial supernatant plus the first wash fraction and was analyzed by electrophoresis in a nondenaturing 10% polyacrylamide–0.5× TBE gel.

Protection of dsRNA by DAI. Radiolabeled dsRNA (354 bp) was bound to immobilized DAI as described above. After the third wash, the beads were washed twice with RNaseIII buffer (100 mM NH_4Cl, 10 mM magnesium acetate, 20 mM Tris-HCl [pH 7.6]) and then incubated with 50 U of RNaseIII per ml for 30 min at 37°C. An equal amount of fresh RNaseIII was added, and the incubation continued for a further 30 min. RNA was isolated from the beads and from the supernatant fractions and analyzed as described above.

Gel retardation assay. Binding reactions (10 μl) were similar to those for kinase assays, except that ATP was omitted and tRNA and BSA were present at 0.1 and 1 mg/ml, respectively. The concentration of labeled dsRNA was 55 ng/ml, and the concentration of DAI (Mono S fraction), immunoaffinity chromatography-purified DAI (14), or p20 (55) was varied. After incubation for 20 min on ice, a dye-glycerol solution was added and the samples were loaded directly onto a 5% polyacrylamide gel (acrylamide: bioacrylamide, 82:1). The gel was cast in 40 mM Tris-glycine buffer and had been prerun for 1 h at 150 V. Radioactivity was detected by autoradiography for approximately 16 h.

RESULTS

Characteristics of synthetic dsRNA. Duplexed RNAs of defined sizes were made by annealing a 358-nt transcript synthesized by T7 RNA polymerase with complementary transcripts of various lengths synthesized by T3 RNA polymerase (Fig. 1A). After digestion of the RNA tails and residual single-stranded RNA, the dsRNAs were purified by electrophoresis in nondenaturing polyacrylamide gels. When analyzed in denaturing conditions (Fig. 1B), the individual strands of the dsRNA molecules were slightly heterogeneous, with chain lengths a few nucleotides longer or shorter than the input single strands as a result of the trimming process. When examined in a nondenaturing gel, however, the dsRNAs migrated as discrete bands, with mobilities similar to those of dsDNA markers (see Fig. 5A, lanes 3 to 9). As expected, the duplexes were sensitive to digestion with RNaseIII, a dsRNA-specific enzyme, but resistant to digestion by single-stranded specific nucleases except after denaturation (data not shown).

Activation and inhibition of DAI. Activation of DAI is accompanied by its autophosphorylation, converting the enzyme from a latent state to a form which can phosphorylate eIF-2α. When the synthetic duplexes were examined for their ability to catalyze autophosphorylation, we found that 23- and 34-bp dsRNAs were only slightly active, 40-bp dsRNA was partly active, and full activity was approached with 55- to 85-bp dsRNAs, which were nearly as active as the very long dsRNA (average size of >2,000 bp) isolated from reovirus virions (Fig. 2A). These results, obtained with essentially flush-ended dsRNA, agree closely with previously reported data obtained by using RNA molecules in which one strand was considerably longer than the other (44): in the earlier study, duplexes shorter than 30 bp were unable to activate DAI, and full activation was obtained with duplexes longer than 65 to 80 bp. The activation of DAI can also be monitored by phosphorylation of eIF-2, the natural substrate of this kinase. In this assay, 15-bp dsRNA was essentially inactive, 34-bp dsRNA was partially active, and 55-bp (or longer) dsRNA was fully active (Fig. 2B). Thus, the slight autophosphorylation of DAI that is catalyzed by the 34-bp duplex is sufficient to permit DAI to phosphorylate its natural substrate weakly. These results are consistent with the findings that the very short (<20 bp) imperfect duplexes found in viral RNAs such as VA RNA (28, 38, 41,

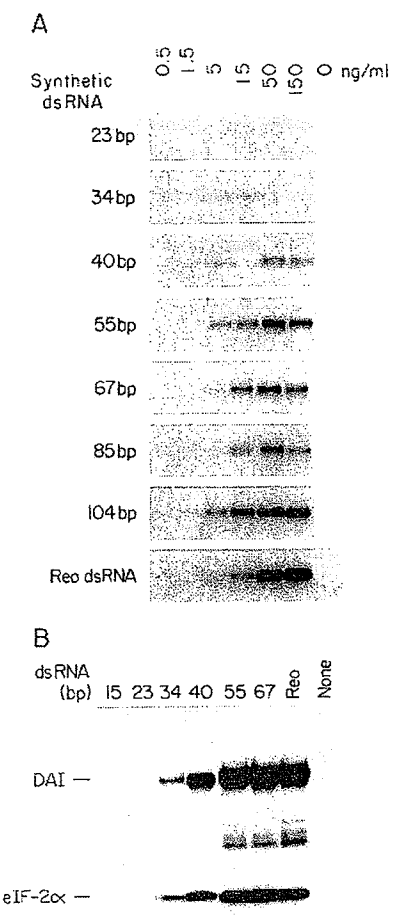

FIG. 2. Activation of DAI by short duplexes. (A) Autophosphorylation of DAI as a function of dsRNA concentration and chain length. Kinase assay reaction mixtures containing synthetic or reovirus dsRNA at the concentrations indicated were analyzed by electrophoresis in SDS-polyacrylamide gels and autoradiography. (B) Phosphorylation of eIF-2. Kinase assay reaction mixtures containing 33 ng of the synthetic dsRNAs indicated per ml or 40 ng of reovirus dsRNA per ml were supplemented with eIF-2 and were analyzed as described in part A. The positions of autophosphorylated DAI and the phosphorylated α subunit of eIF-2 are marked.

42, 48), EBER (4, 7) and TAR RNA (18) are insufficient to cause DAI activation.

From experiments with tailed duplexes, it was concluded that short dsRNAs which are incapable of activating DAI may nevertheless block the activation of DAI by longer dsRNAs (44). Figure 3A shows that at high concentration a flush-ended 23-bp dsRNA inhibited the activation of DAI by reovirus dsRNA. In this respect, short dsRNA resembles longer dsRNA molecules which block DAI activation at high concentrations, although the mechanisms might be different. Figure 3B demonstrates that long dsRNA (approximately 3,000 bp) isolated from *P. chrysogenum* activates DAI at concentrations of up to 1 μg/ml and prevents activation at 10 μg/ml, as expected from the bell-shaped activation curve.

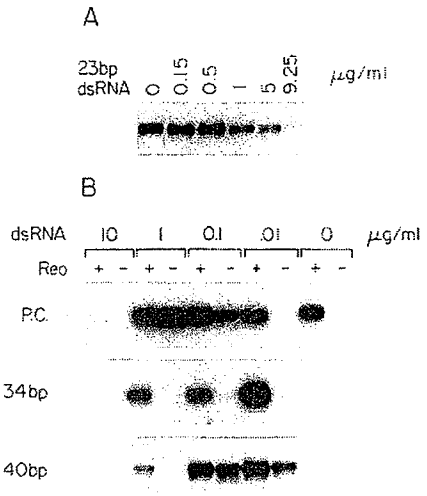

FIG. 3. Inhibition of DAI activation by high concentrations of dsRNA. (A) Inhibition of DAI autophosphorylation by 23-bp dsRNA. Kinase assay reaction mixtures contained 20 ng of reovirus dsRNA per ml and the indicated concentrations of the 23-bp dsRNA. (B) Effect of duplexes of various lengths on DAI autophosphorylation in the presence and absence of an activator dsRNA. Kinase assay reaction mixtures containing or lacking reovirus dsRNA (40 ng/ml) were supplemented with synthetic 34- or 40-bp dsRNAs or with *P. chrysogenum* dsRNA (P.C.) at the concentrations indicated. Autophosphorylation of DAI was assessed as described for Fig. 2.

Short duplexes, such as the 34-bp dsRNA (Fig. 3B), activate DAI only weakly and also become inhibitory at approximately 10 μg/ml. Similar results were obtained with bacteriophage f2 sus3 dsRNA (approximately 30 to 50 bp long [22]) and the 23-bp synthetic duplexes (data not shown), while the 40-bp dsRNA gave a response intermediate between that of the longer and shorter duplexes because of its significant ability to activate DAI (Fig. 3B). Single-stranded RNA is not inhibitory (data not shown). These results confirm that the enzyme is activated by relatively long dsRNA and can interact with short duplexes in a nonproductive way.

RNA size dependence of DAI binding. The existence of opposing effects of dsRNA on DAI activity makes it important to address directly the binding of this ligand to the protein. We examined the interactions between DAI and dsRNA, using three assays which explore different aspects of the process: nitrocellulose filter binding, binding to an affinity matrix, and gel retardation. Figure 4A and B illustrates the binding of single-stranded RNA and dsRNA as a function of chain length in the filtration assay. Duplexes with sizes of 15 and 23 bp did not bind detectably, whereas 34- and 40-bp duplexes bound weakly. Longer molecules bound with increasing efficiency, and 85- or 104-bp dsRNA bound as efficiently as 354-bp molecules. Single-stranded molecules bound only very weakly, except for the 354-nt molecule, which may be able to form a small amount of secondary structure. The binding of dsRNAs to DAI was also measured as a function of ligand concentration (Fig. 4C and D). The efficiency with which short duplexes bound to DAI did not improve at subsaturating RNA concentrations, suggesting that the affinity of the enzyme is low for molecules with sizes

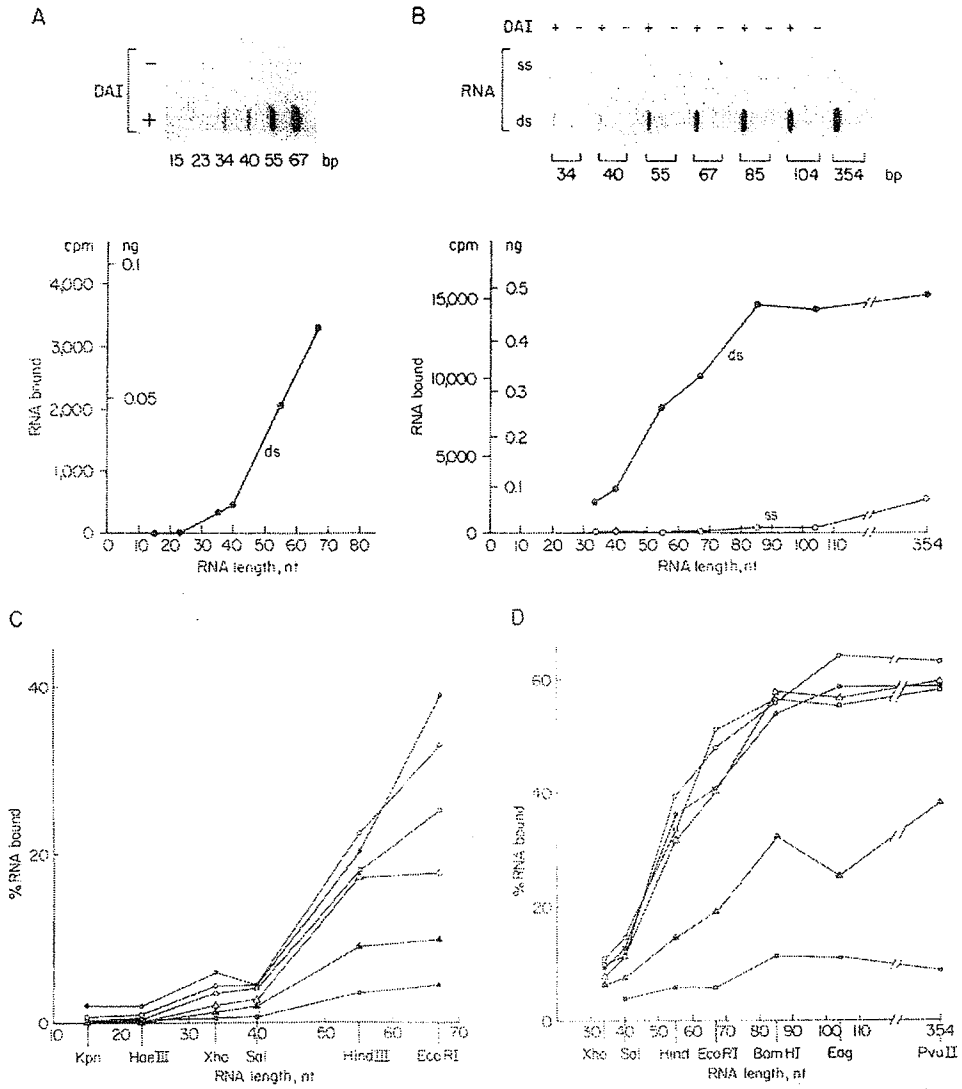

FIG. 4. Size dependence of dsRNA binding to DAI. (A and B) Nitrocellulose filter-binding assay for the binding of single-stranded RNA and dsRNAs. Autoradiograms of the membrane are shown (top panel). Quantitation of RNA binding (bottom panel) was obtained by scintillation counting or scanning of the individual filter bands and subtraction of the background value (lanes labeled −DAI) from the signal radioactivity (lanes labeled +DAI). ●, dsRNA (ds); ○, single-stranded RNA (ss). (C and D) Concentration and size dependence of dsRNA-binding efficiency. The percentage of the input dsRNA that was retained on the filter in the presence of DAI was quantified at various dsRNA concentrations of 1,000 (■), 330 (▲), 100 (△), 33 (□), 10 (○), and 3.3 (●) ng/ml, respectively.

of 40 bp or less and that the affinity increases uniformly as the chain length is increased, reaching a maximum at 85 bp. These data agree closely with the dependence of activation and inhibition on dsRNA chain length (Figs. 2 and 3) and are consistent with a model which equates activation with stable dsRNA binding. Duplexes shorter than 30 to 40 bp bind weakly and cannot activate although they inhibit activation; longer duplexes (40 to 85 bp) bind with increasing stability and their ability to activate the enzyme increases concomitantly; beyond this length, the efficiency of binding and activation remains unchanged.

Binding and protection of dsRNA. One interpretation of these observations is that the dsRNA binding site in DAI accommodates up to ~85 bp of duplex but can bind shorter duplexes less stably, down to ~30 bp. To test this interpretation we employed DAI immobilized on antibody-Sepharose beads. First, to define the minimum size of dsRNA that can bind to the enzyme, a mixture of dsRNA molecules was partially digested with RNaseIII to generate a collection of duplex molecules with a broad size distribution. This collection was allowed to bind to the immobilized DAI, and the beads were washed to remove nonspecifically adsorbed dsRNA. Figure 5A displays the DAI-bound dsRNA (lane 1) and the unbound RNA that remained in the supernatant (lane

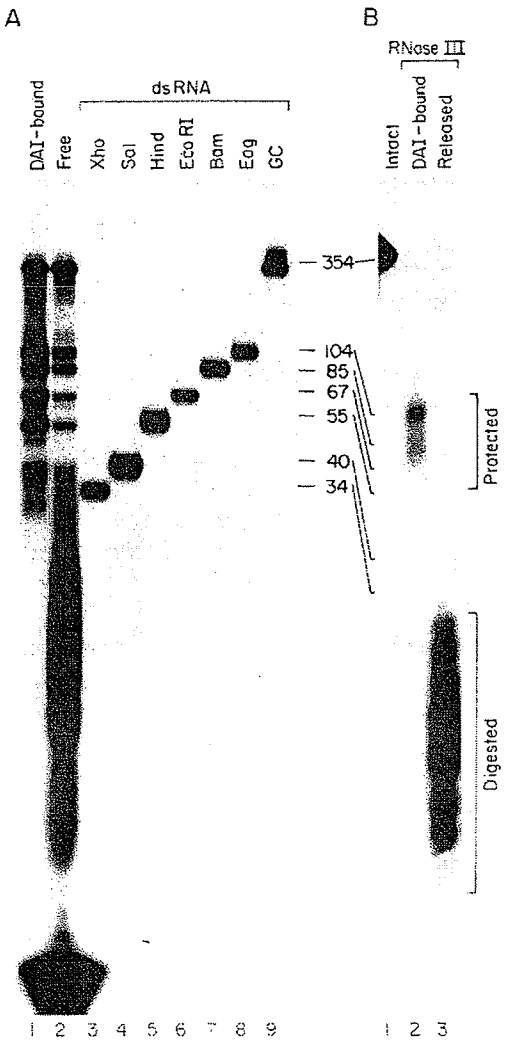

FIG. 5. Binding and protection of dsRNA fragments by DAI. (A) Binding of randomly sized dsRNA fragments. A mixture of fragments (approximately 10 to 350 bp) was incubated with DAI attached to Sepharose beads. Unbound RNA was separated from the beads by centrifugation and washing. Equal fractions of the DAI-bound (lane 1) and unbound (lane 2) dsRNA were resolved in a nondenaturing polyacrylamide gel and detected by autoradiography. Markers included discrete dsRNA fragments with sizes of 34 to 104 bp (lanes 3 to 8), denoted by the restriction site designation used in their synthesis (Fig. 1A), and 354 bp (lane 9), denoted GC for the plasmid used in its synthesis (pGEM.GC). (B) Protection of DAI-bound dsRNA from digestion by RNaseIII. Discrete 354-bp dsRNA (lane 1) was bound to DAI attached to Sepharose beads, and unbound RNA was removed. The beads were exhaustively incubated with RNaseIII, and the released RNA fragments were collected. Equal fractions of the released RNA (lane 3) and the RNA that remained associated with the beads (lane 2) were resolved as described for panel A.

2). Comparison with dsRNA markers (lanes 3 to 9) and with an RNA sequence ladder (data not shown), indicated that the cutoff for binding was at approximately 28 bp, in good agreement with results obtained in the nitrocellulose filter-binding assay. Moreover, visual inspection of the autoradiogram suggested that dsRNA with a size of 28 to 40 bp bound less efficiently than longer duplexes.

Next, we conducted a protection experiment to determine the length of dsRNA that is shielded by DAI from nuclease attack. Intact 354-bp dsRNA was bound to DAI immobilized on antibody-Sepharose beads, and the excess unbound dsRNA was removed. The bound dsRNA was digested by incubating the beads with RNaseIII to trim off regions of duplex that were not protected by DAI. Figure 5B, lane 3, shows that the released dsRNA had been reduced to fragments of approximately 10 to 20 bp as expected (57), whereas the bulk of the DAI-associated material (lane 2) ranged in size from approximately 60 to 120 bp, with a substantial concentration in the longer-size class (approximately 100 to 120 bp). Assuming that RNaseIII leaves 15 bp of dsRNA protruding on each side, we deduce that DAI associates with 30 to 90 bp of dsRNA. Taking 110 bp as the modal length of the protected fragments, it appears that about 80 bp of duplex interact directly with the enzyme, roughly the length of dsRNA that gives maximal binding in the nitrocellulose filter assay. The length of the protected fragment was not altered at relatively high concentrations of dsRNA (up to 1 μg/ml; data not shown), conditions which would be expected to disfavor oligomerization of DAI on the dsRNA. These findings support the view that the dsRNA site extends for ~80 bp and that shorter molecules bind with lesser affinity, provided that they are at least 28 bp long.

DAI-dsRNA complexes. To characterize the interactions more directly, we examined complexes formed between DAI and dsRNAs in a gel retardation assay (Fig. 6A). No complexes were observed with 15- or 23-bp duplexes (data not shown), but longer dsRNAs formed complexes with increasing efficiency. Four series of complexes were distinguishable (bands I to IV). Their relative abundance was principally a function of RNA chain length, with a lesser dependence on DAI concentration. On the basis of their behavior, the complexes seem to fall into two families. One family, containing the more slowly moving bands I and II, forms preferentially with duplexes of less than optimal length (34 to 67 bp) in binding and activation assays. The second family, containing the faster moving bands III and IV, forms preferentially with longer duplexes (≥85 bp), which are fully active in binding and activating DAI.

Band I was the most prominent complex with 55- and 67-bp duplexes but was barely detectable with longer or shorter duplexes. It was formed at low DAI concentrations and seemed to be converted to band II at elevated DAI concentrations. Indeed, regardless of chain length, complex II was seen only at high DAI concentrations. With 34- and 40-bp duplexes, the only detectable complexes appeared to migrate in band II. Formation of complex II increased as the chain length was extended, reaching maximal levels with the 67-bp duplex and declining as the chain length was extended further, to 85 and 104 bp.

The most abundant complexes, formed with 85- and 104-bp duplexes, migrated in band III. This band was also visible with 67-bp and perhaps 55-bp dsRNAs. As chain length increased, complex III was formed at progressively lower concentrations of DAI. It also seemed to decrease slightly at high DAI concentrations. Band IV displayed a pattern similar to that of band III but was always less

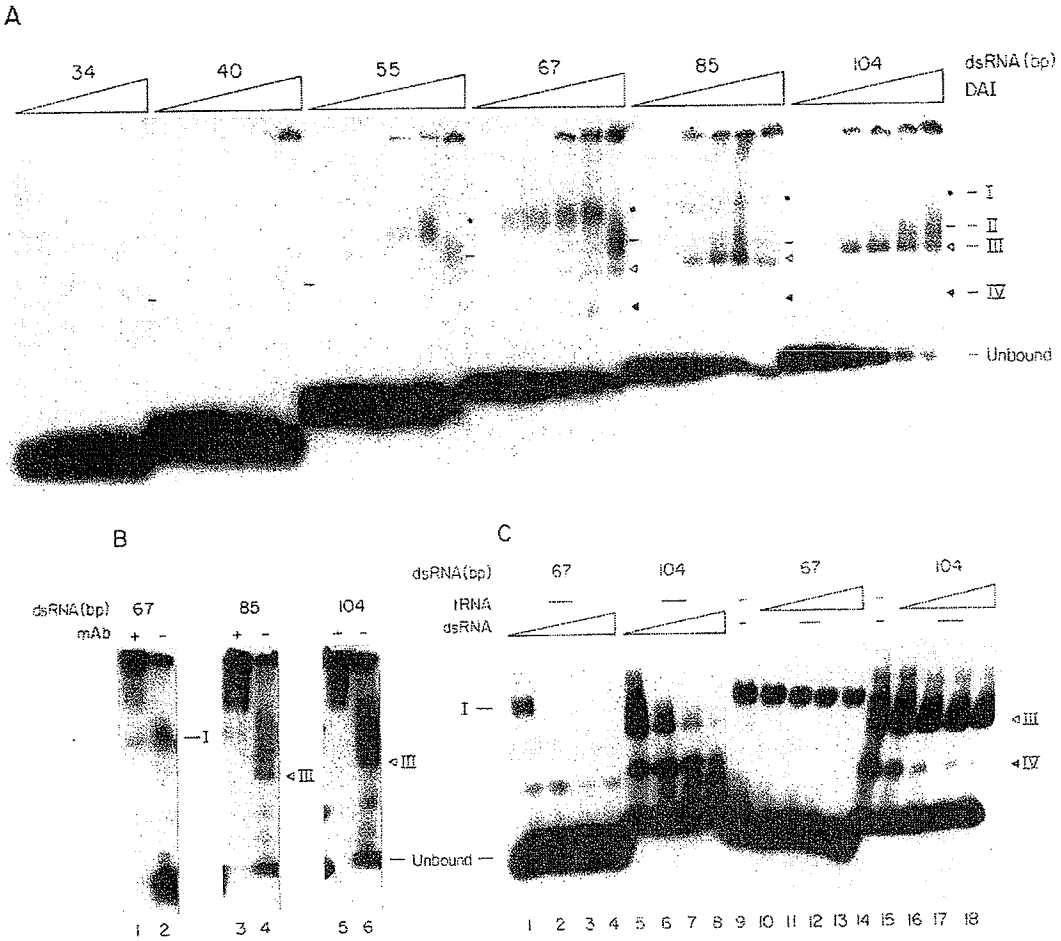

FIG. 6. Gel-shift analysis with DAI. (A) Dependence on dsRNA size and DAI concentration. dsRNAs with sizes of 34 to 104 bp were incubated with various amounts of DAI (0, 0.05, 0.1, 0.25, 0.5, and 1.0 μl from left to right, symbolized by the wedges) purified to the Mono S stage. The resultant complexes were separated by electrophoresis in nondenaturing conditions and detected by autoradiography. The positions of complexes I (●), II (—), III (◁), and IV (◀) and of the free dsRNA fragments are marked in each panel. (B) Shifts with essentially homogeneous DAI and antibody supershift. dsRNA with a size of 67 bp (lanes 1 and 2), 85 bp (lanes 3 and 4), or 104 bp (lanes 5 and 6) was incubated with 1 μl of DAI purified by immunoaffinity chromatography in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of 1 μl of monoclonal antibody to DAI. (C) Competition assays. Standard reaction mixtures (lanes 9 and 14) contained DAI (Mono S fraction) and 100 μg of tRNA per ml; *P. chrysogenum* dsRNA (0.25, 0.5, 0.75, and 1 μg/ml; lanes 1 to 4 and 5 to 8) or additional calf liver tRNA (100, 200, 300 and 400 μg/ml; lanes 10 to 13 and 15 to 18) was added as indicated. ^{32}P-labeled dsRNA (67 bp [lanes 1 to 4 and 9 to 13] or 104 bp [lanes 5 to 8 and 14 to 18]) was present at 55 ng/ml. The wedges symbolize increasing concentrations from left to right; — indicates absence of the RNA.

abundant. Both of these bands correlate well with full enzyme activity.

Very similar patterns of bands were formed with an essentially homogeneous preparation of DAI purified by immunoaffinity chromatography (Fig. 6B), and the gel-shift activity cosedimented with kinase activity through a glycerol gradient (29) (data not shown). Furthermore, as seen in Fig. 6B, all of the complexes were "supershifted" to forms with slower mobility by addition of monoclonal antibody directed against DAI. The antibody did not produce a gel shift on its own (i.e., in the absence of DAI), but it appeared to stabilize DAI-dsRNA complexes so that less probe remained in free form. These experiments verified that bands I to IV all contain DAI. Competition experiments demonstrated that the most prominent complexes, band I with 67-bp dsRNA and band III with 104-bp dsRNA, were resistant to the presence of excess tRNA but were sensitive to unlabeled *P. chrysogenum* dsRNA competitor (Fig. 6C). The complexes formed with the 104-bp dsRNA were more resistant to competition than those formed with 67-bp dsRNA, as expected from the higher affinity of DAI for longer duplexes (Fig. 4). For some of the minor bands (e.g., band IV), competition was more effective with tRNA at very high concentrations than with dsRNA at moderate concentrations

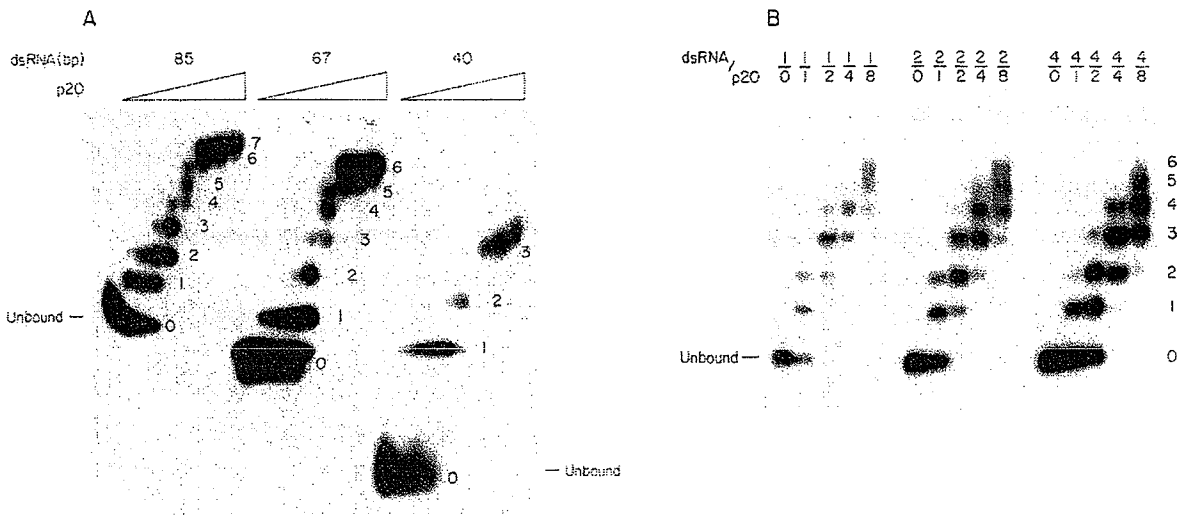

FIG. 7. Gel shift analysis with the p20 polypeptide. (A) Dependence on dsRNA size. Reaction mixtures contained dsRNA of 40, 67, or 85 bp as indicated and 0, 0.032, 0.063, 0.125, 0.25, 0.5, 2.5, 5, or 10 μg/ml of p20 (increasing from left to right as symbolized by the wedges). The number of p20 units in each band is indicated on the right. (B) Dependence on dsRNA and p20 concentration. Assay mixtures contained 85-bp dsRNA at a concentration of 55, 110, or 220 μg/ml and p20 at a concentration of 0, 63, 125, 250, or 500 ng/ml. Concentrations increase from left to right. The relative concentrations of these two components are indicated above the autoradiogram, and the number of p20 units in each band is marked on the side.

for reasons that are presently unclear. Neither single-stranded DNA nor dsDNA was an effective competitor (data not shown). These results demonstrate that the most prominent complexes are dsRNA and DAI specific and that there is a rather abrupt change in electrophoretic mobility when the dsRNA reaches the size for optimal binding and activation (approximately 80 bp).

Minimal binding site. The ability of short duplexes (<30 bp) to inhibit DAI activation implies that they interact with the enzyme, even though their binding is not sufficiently stable to be detected by the assays used to this point. To determine the minimal length of duplex that can interact with DAI, we employed the close-packing method for estimating the number of protein molecules that can bind to duplexes with known sizes. For this purpose, we used a truncated version of DAI, p20, comprising the N-terminal 184 amino acids which we and others have determined to contain the RNA-binding domain of the protein (16, 25, 39, 50). The numbers of p20 molecules binding to a given dsRNA were estimated from gel-shift assays conducted at increasing concentrations of the protein. As seen in Fig. 7A, a series of complexes was formed, reaching a maximum at the highest protein levels. The number of complexes increased with increasing dsRNA chain length as follows: 40 bp, three bands; 67 bp, six bands; 85 bp, seven bands. Assuming that each shifted band corresponds to the binding of a p20 molecule, these data imply that the minimum binding site is about 11 bp, equivalent to a single turn of A-form RNA helix.

The appearance of a ladder of bands with p20 suggested that there may be differences between the binding of this fragment and intact DAI to dsRNA. Further experiments showed that the formation of the p20 complexes is specific in that tRNA does not compete (data not shown). To rule out the possibility that the p20 banding patterns are due to a concentration-dependent protein oligomerization that is independent of dsRNA, we conducted band-shift assays at increasing dsRNA concentrations. Figure 7B shows that more p20 protein is required to achieve a given gel shift at higher concentrations of dsRNA (compare, for example, the amount of p20 needed to complex all of the dsRNA in the reactions). This indicates that oligomerization depends on the presence of dsRNA and is due to the formation of a series of protein-RNA complexes rather than to preformed protein-protein aggregates. The banding pattern was also influenced by the absolute concentration of p20 and dsRNA, however, as can be seen by comparing lanes with equal ratios of dsRNA to p20 (such as 1/1, 2/2, and 4/4). The shift to larger complexes at higher concentrations could merely reflect the concentration dependence of the reaction according to the law of mass action, or it could imply that p20 complexes are stabilized by protein-protein interactions when p20 monomers are bound adjacently on dsRNA. In the latter case, stabilizing protein-protein interactions would provide an explanation for the apparent paradox that DAI binds efficiently only to duplexes of longer than 30 bp but can bind to as little as a single helical turn of dsRNA.

DISCUSSION

Although the existence of DAI has been known for many years and its activation by a variety of polynucleotides has been studied intensively, an understanding of the enzyme's regulation has remained elusive. The kinase is activated by autophosphorylation in the presence of dsRNA. This response exhibits a number of unusual features: first, activation is prevented by high concentrations of dsRNAs which activate the kinase at low concentrations; second, short RNA duplexes fail to activate DAI at any concentration but prevent activation at elevated concentrations; third, highly structured single-stranded RNAs of viral origin also fail to activate DAI but can block activation by authentic, long

dsRNA. To illuminate the interactions between dsRNA and DAI, we generated a series of short RNA duplexes and studied directly their binding to the enzyme as well as their effects on its activity. The results correlate activation with the formation of stable complexes with a characteristic electrophoretic mobility and suggest a model that is compatible with the emerging understanding of DAI structure.

Our results are most consistent with the view that DAI possesses a single effective site for dsRNA, capable of accommodating approximately 80 bp of duplex. Two observations support this conclusion most strongly. First, as the length of the dsRNA ligand is increased, maximal binding is attained at this size and longer molecules bind no more efficiently, and second, the kinase protects this length of duplex from digestion by nuclease. Shorter duplexes, down to a lower limit of approximately 30 bp, bind with steadily decreasing efficiency while duplexes with lengths of less than 30 bp are unable to form a stable complex with DAI under normal conditions. Nonetheless, since such very short duplexes block the activation of DAI, we assume that at high concentrations they form transient interactions which prevent DAI activation. Likewise, other polynucleotides, such as RNA-DNA hybrids and partially methylated dsRNA duplexes that fail to activate the kinase, as well as long dsRNAs that can activate DAI, share this property of inhibiting kinase activation at high concentrations. The nature of these inhibitory interactions is unclear, and it remains to be seen whether viral effectors such as VA RNA, EBER, and TAR RNA function in the same way as short duplexes or whether they interact in a distinct fashion to block DAI activation. Preliminary data indicate that the sites for VA RNA and dsRNA are overlapping but perhaps not congruent (16).

How do these functional observations relate to the structure of the enzyme? DAI possesses two RNA-binding elements in its N-terminal domain (12, 16, 25, 39, 50). Each element contains an RNA-binding motif which is rich in basic amino acids and is predicted to form an α-helical structure (16, 39). Both elements are required for efficient binding of RNA, and they appear to cooperate to form a single bivalent site which optimally extends over approximately 80 bp of duplex. Since the RNA binding domain of DAI, expressed as the p20 protein, is able to interact with as little as 11 bp of dsRNA, we speculate that each element interacts with a single helical turn and that optimal binding occurs when these two turns are separated by about five intervening helical turns. In this complex, the entire span of approximately 80 bp is protected by DAI against attack by the dsRNA-specific nuclease RNaseIII. With this model, interactions with shorter dsRNA molecules entail increasing strain on the enzyme, accompanied by decreasing affinity, such that it becomes impossible for both elements to bind when there is less than one intervening helical turn (at approximately 33 bp). Evidently, monovalent complexes can also be formed at high ratios of enzyme to RNA as in the p20 gel-shift experiments: these complexes presumably involve only the stronger RNA binding region (region 1 [16]) and allow the protein to pack onto the RNA to a density of one molecule per helical turn.

According to this model, activation of the enzyme requires bivalent dsRNA binding which becomes detectable at approximately 30 bp and is most stable when the duplex is at least 80 bp long. Correlating with the formation of the most stable complexes is a shift in their mobility in the gel retardation assay. The predominant complex formed with dsRNA with a length of ≥85 bp is band III, which moves faster than the predominant complex formed with shorter dsRNA (band I). We considered the possibility that longer duplexes might be able to bind more DAI molecules than shorter duplexes, but because DAI is a basic protein (pI 8.6), it is unlikely that the acceleration in gel mobility that occurs between 67 and 85 bp with the shift from complex I to complex III is due to the binding of a second DAI molecule to a DAI-dsRNA complex. Therefore, we argue that the faster migration is probably due to a conformational change in the dsRNA or the DAI-dsRNA complex which leads to compaction and increased electrophoretic mobility. Compaction could result from relief of the distortion in DAI that occurs when the two binding elements can interact with optimally spaced sites on dsRNA. Alternatively, it could be accomplished if the RNA were bent or wrapped around the enzyme once it had filled the entire site. If this explanation is correct, it seems that the duplex must be continuous since an elevated concentration of 40-bp molecules does not have the same effect on binding or activation as an 80-bp duplex. The minor complexes, II and IV, seem to be related to complexes I and III, respectively, but display increased sensitivity to competition with tRNA. They are unlikely to represent the addition of a second molecule of DAI to a DAI-dsRNA complex because of the large retardation effect that this would be expected to have on electrophoretic mobility and there are few clues as to their structure or significance at present.

The proposal that DAI contains a single bipartite RNA-binding site provides an alternative to the two previous models for DAI activation, neither of which is readily compatible with the results presented here. The gel-shift data could be interpreted in terms of the model in which DAI possesses two distinct sites for dsRNA binding (15, 37), a high-affinity site for activation and a low-affinity inhibitory site, if it were supposed that duplexes with sizes of ≤67 bp bind at the inhibitory site whereas longer duplexes bind at the activating site. The resultant complexes could have significantly different mobilities. However, the apparent affinities for these duplexes are not greatly different in the binding assays shown here, so the postulate that the activation site is of much higher affinity than the inhibitory site is not satisfied. Moreover, 40- to 67-bp duplexes have significant ability to activate the enzyme. The data could also be interpreted in terms of the model that DAI is activated when two molecules bind to a single molecule of dsRNA (37, 49). On this basis, 80 bp would be the length of duplex required to span the RNA-binding sites of two DAI monomers. Each monomer would interact with 30 to 40 bp of dsRNA, and the complex would be stabilized by cooperative interactions between the protein molecules. The monotonous increase in binding efficiency with a chain length between 30 and 85 bp argues against this model, as does our failure to obtain protection of shorter RNA fragments at high ratios of DAI to dsRNA. Also, with this model, it is difficult to explain the multiplicity of complexes observed in band-shift experiments: in particular, longer duplexes or higher DAI concentrations would be expected to give rise to slower complexes, contrary to observations.

In summary, the data presented here suggest that DAI interacts with as little as 11 bp (one helical turn) of dsRNA, but activation is associated with the formation of a stable DAI-dsRNA complex. The formation of such a complex requires at least 30 bp of duplex (about three turns) and probably takes place when both of the enzyme's RNA-binding motifs are engaged with the ligand. Complex formation is optimal with dsRNA containing at least 80 bp (seven

to eight turns) and is apparently accompanied by a conformational change in the complex. We speculate that the bivalent interaction with dsRNA or the conformational change itself is critical for enzyme autophosphorylation and activation. With this model, short dsRNAs would be expected to block activation because they can interact with only one RNA-binding motif. Specific inhibitors of DAI activation, such as VA RNA, may also bind to one motif, or alternatively they may bind to both motifs but in such a way as to interfere with the conformation of the enzyme. Similarly, long dsRNAs that activate the enzyme at low concentrations might block activation at high concentrations because the two binding motifs form complexes with separate RNA duplexes, thereby precluding the requisite conformational change. While this is a satisfying explanation, there are alternatives which also fit the available facts. For example, since autophosphorylation appears to be intermolecular (29, 37), it is also possible that DAI serves as a phosphate acceptor only when it is not bound to dsRNA, a situation which would obtain at low or moderate concentrations of dsRNA. Consistent with this view, a truncated form of DAI that lacks the RNA binding site can still be phosphorylated by intact DAI (unpublished data). It is clear that further investigation will be required to establish the nature of the coupling between dsRNA binding and kinase activation: such studies are in progress.

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REFERENCES

1. **Baglioni, C., and P. A. Maroney.** 1981. Inhibition of double-stranded ribonucleic acid activated protein kinase and 2',5'-oligo(adenylic acid) polymerase by ethidium bromide. Biochemistry **20**:758–762.
2. **Baglioni, C., M. A. Minks, and E. DeClercq.** 1981. Structural requirements of polynucleotides for the activation of (2'-5')A_n polymerase and protein kinase. Nucleic Acids Res. **9**:4939–4950.
3. **Berry, M. J., G. S. Knutson, S. R. Laskey, S. M. Munemitsu, and C. E. Samuel.** 1985. Purification and substrate specificities of the double-stranded RNA-dependent protein kinase from untreated and interferon-treated mouse fibroblasts. J. Biol. Chem. **260**:11240–11247.
4. **Bhat, R. A., and B. Thimmappaya.** 1985. Construction and analysis of additional adenovirus substitution mutants confirm the complementation of VAI RNA function by two small RNAs encoded by Epstein-Barr virus. J. Virol. **56**:750–756.
5. **Bischoff, J. R., and C. E. Samuel.** 1989. Mechanism of interferon action. Activation of the human P1/eIF-2α protein kinase by individual reovirus s-class mRNAs: s1 mRNA is a potent activator relative to s4 mRNA. Virology **172**:106–115.
6. **Chong, K. L., L. Feng, K. Schappert, E. Meurs, T. F. Donahue, J. D. Friesen, A. G. Hovenessian, and B. R. G. Williams.** 1992. Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator *GCN2*. EMBO J. **11**: 1553–1562.
7. **Clarke, P. A., N. A. Sharp, and M. J. Clemens.** 1990. Translational control by the Epstein-Barr virus small RNA EBER-1. Reversal of the double-stranded RNA-induced inhibition of protein synthesis in reticulocyte lysates. Eur. J. Biochem. **193**:635–641.
8. **deBenedetti, A., and C. Baglioni.** 1984. Inhibition of mRNA binding to ribosomes by localized activation of dsRNA-dependent protein kinase. Nature (London) **311**:79–81.
9. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell **68**:585–596.
10. **Dubois, M.-F., J. Galabru, P. Lebon, B. Safer, and A. G. Hovanessian.** 1989. Reduced activity of the interferon-induced double-stranded RNA-dependent protein kinase during a heat shock stress. J. Biol. Chem. **264**:12165–12171.
11. **Farrell, P. J., K. Balkow, T. Hunt, and R. J. Jackson.** 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. Cell **11**:187–200.
12. **Feng, G.-S., K. Chong, A. Kumar, and B. R. G. Williams.** 1992. Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. Proc. Natl. Acad. Sci. USA **89**:5447–5451.
13. **Galabru, J., and A. Hovanessian.** 1985. Two interferon-induced proteins are involved in the protein kinase complex dependent on double-stranded RNA. Cell **43**:685–694.
14. **Galabru, J., and A. Hovanessian.** 1987. Autophosphorylation of the protein kinase dependent on double-stranded RNA. J. Biol. Chem. **262**:15538–15544.
15. **Galabru, J., M. G. Katze, N. Robert, and A. G. Hovanessian.** 1989. The binding of double-stranded RNA and adenovirus VAI RNA to the interferon-induced protein kinase. Eur. J. Biochem. **178**:581–589.
16. **Green, S. R., and M. B. Mathews.** Submitted for publication.
17. **Grodberg, J., and J. J. Dunn.** 1988. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. J. Bacteriol. **170**:1245–1253.
18. **Gunnery, S., A. P. Rice, H. D. Robertson, and M. B. Mathews.** 1990. Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **87**:8687–8691.
19. **Hershey, J. W. B.** 1989. Protein phosphorylation controls translation rates. J. Biol. Chem. **264**:20823–20826.
20. **Hershey, J. W. B.** 1991. Translational control in mammalian cells. Annu. Rev. Biochem. **60**:717–755.
21. **Hovanessian, A. G.** 1989. The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK. J. Interferon Res. **9**:641–647.
22. **Hunter, T., T. Hunt, R. J. Jackson, and H. D. Robertson.** 1975. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. J. Biol. Chem. **250**:409–417.
23. **Judware, R., and R. Petryshyn.** 1991. Partial characterization of a cellular factor that regulates the double-stranded RNA-dependent eIF-2α kinase in 3T3-F442A fibroblasts. Mol. Cell. Biol. **11**:3259–3267.
24. **Katze, M. G., B. M. Detjen, B. Safer, and R. M. Krug.** 1986. Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral messenger RNAs. Mol. Cell. Biol. **6**:1741–1750.
25. **Katze, M. G., M. Wambach, M.-L. Wong, M. Garfinkel, E. Meurs, K. Chong, B. R. G. Williams, A. G. Hovanessian, and G. N. Barber.** 1991. Functional expression and RNA binding analysis of the interferon-induced, double-stranded RNA-activated, 68,000-M_r protein kinase in a cell-free system. Mol. Cell. Biol. **11**:5497–5505.
26. **Kaufman, R. J., and P. Murtha.** 1987. Translational control mediated by eukaryotic initiation factor-2 is restricted to specific mRNAs in transfected cells. Mol. Cell. Biol. **7**:1568–1571.
27. **Kimchi, A., A. Zilberstein, A. Schmidt, L. Shulman, and M. Revel.** 1979. The interferon-induced protein kinase PK-i from mouse L cells. J. Biol. Chem. **254**:9846–9853.
28. **Kitajewski, J., R. J. Schneider, B. Safer, S. M. Munemitsu, C. E. Samuel, B. Thimmappaya, and T. Shenk.** 1986. Adenovirus VAI RNA antagonizes the antiviral action of the interferon by preventing activation of the interferon-induced eIF-2α kinase. Cell **45**:195–200.

29. **Kostura, M., and M. B. Mathews.** 1989. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. Mol. Cell. Biol. **9**:1576–1586.
30. **Lasky, S. R., B. L. Jacobs, and C. E. Samuel.** 1982. Characterization of the sites of phosphorylation in the interferon-induced phosphoprotein P1 from mouse fibroblasts: evidence for two forms of P1. J. Biol. Chem. **257**:11087–11093.
31. **Laurent, A. G., B. Krust, J. Galabru, J. Svab, and A. G. Hovanessian.** 1985. Monoclonal antibodies to an interferon-induced M_r 68,000 protein and their use for the detection of double-stranded RNA-dependent protein kinase in human cells. Proc. Natl. Acad. Sci. USA **82**:4341–4345.
32. **Lebleu, B., G. C. Sen, S. Shaila, B. Cabrer, and P. Lengyel.** 1976. Interferon, double-stranded RNA, and protein phosphorylation. Proc. Natl. Acad. Sci. USA **73**:3107–3111.
33. **Lenz, J. R., and C. Baglioni.** 1978. Inhibition of protein synthesis by double-stranded RNA and phosphorylation of initiation factor, eIF-2. J. Biol. Chem. **253**:4219–4223.
34. **Levin, D., and I. M. London.** 1978. Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2. Proc. Natl. Acad. Sci. USA **75**:1121–1125.
35. **Levin, D. H., R. Petryshyn, and I. M. London.** 1980. Characterization of double-stranded-RNA-activated kinase that phosphorylates α subunit of eukaryotic initiation factor 2 (eIF-2α) in reticulocyte lysates. Proc. Natl. Acad. Sci. USA **77**:832–836.
36. **Mathews, M. B.** 1991. Control of translation in adenovirus infected cells. Enzyme **44**:250–264.
37. **Mathews, M. B., S. Gunnery, L. Manche, K. H. Mellits, and T. Pe'ery.** 1990. Control of protein synthesis by RNA regulators. NATO ASI (Adv Sci Inst) Ser H Cell Biol. **49**:377–388.
38. **Mathews, M. B., and T. Shenk.** 1991. Adenovirus virus-associated RNA and translational control. J. Virol. **65**:5657–5662.
39. **McCormack, S. J., D. C. Thomis, and C. E. Samuel.** 1992. Mechanism of interferon action: identification of a RNA binding domain within the N-terminal region of the human RNA-dependent P1/eIF-2α protein kinase. Virology **188**:47–56.
40. **Mellits, K. H., M. Kostura, and M. B. Mathews.** 1990. Interaction of adenovirus VA RNA_I with the protein kinase DAI: non-equivalence of binding and function. Cell **61**:843–852.
41. **Mellits, K. H., and M. B. Mathews.** 1988. Effects of mutations in stem and loop regions on the structure and function of adenovirus VA RNA_I. EMBO J. **7**:2849–2859.
42. **Mellits, K. H., T. Pe'ery, L. Manche, H. D. Robertson, and M. B. Mathews.** 1990. Removal of double-stranded contaminants from RNA transcripts: synthesis of adenovirus VA RNA_I from a T7 vector. Nucleic Acids Res. **18**:5401–5406.
43. **Meurs, E., K. Chong, J. Galabru, N. S. B. Thomas, I. M. Kerr, B. R. G. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62**: 379–390.
44. **Minks, M. A., D. K. West, S. Benvin, and C. Baglioni.** 1979. Structural requirements of double-stranded RNA for the activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J. Biol. Chem. **254**:10180–10183.
45. **Minks, M. A., D. K. West, S. Benvin, J. J. Greene, P. O. P. Ts'o, and C. Baglioni.** 1980. Activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells by 2′-O-methylated poly (inosinic acid) · poly (cytidylic acid). J. Biol. Chem. **255**:6403–6407.
46. **Ochoa, S.** 1983. Regulation of protein synthesis initiation in eukaryotes. Arch. Biochem. Biophys. **223**:325–349.
47. **O'Malley, R. P., R. F. Duncan, J. W. B. Hershey, and M. B. Mathews.** 1989. Modification of protein synthesis initiation factors and shut-off of host protein synthesis in adenovirus-infected cells. Virology **168**:112–118.
48. **O'Malley, R. P., T. M. Mariano, J. Siekierka, and M. B. Mathews.** 1986. A mechanism for the control of protein synthesis by adenovirus VA RNA_I. Cell **44**:391–400.
49. **O'Malley, R. P., T. M. Mariano, J. Siekierka, W. C. Merrick, P. A. Reichel, and M. B. Mathews.** 1986. The control of protein synthesis by adenovirus VA RNA. Cancer Cells **4**:291–301.
50. **Patel, R. C., and G. C. Sen.** 1992. Identification of the double-stranded RNA-binding domain of the human interferon-inducible protein kinase. J. Biol. Chem. **267**:7671–7676.
51. **Pestka, S., J. A. Langer, K. C. Zoon, and C. E. Samuel.** 1987. Interferons and their actions. Annu. Rev. Biochem. **56**:727–777.
52. **Petryshyn, R., J.-J. Chen, and I. M. London.** 1984. Growth-related expression of a double-stranded RNA-dependent protein kinase in 3T3 cells. J. Biol. Chem. **259**:14736–14742.
53. **Rice, A. R., M. Kostura, and M. B. Mathews.** 1989. Identification of a 90 KD polypeptide which associates with adenovirus VA RNA_I and is phosphorylated by the double-stranded RNA dependent protein kinase. J. Biol. Chem. **264**:20632–20637.
54. **Samuel, C. E.** 1991. Antiviral actions of interferon: interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183**:1–11.
55. **Schmedt, C., S. R. Green, and M. B. Mathews.** Unpublished data.
56. **Schneider, R. J., and T. Shenk.** 1987. Impact of virus infection on host cell protein synthesis. Annu. Rev. Biochem. **56**:317–332.
57. **Schweitz, H., and J. P. Ebel.** 1971. A study of the mechanism of action of E. coli ribonuclease III. Biochimie **53**:585–593.
58. **Sen, G. C., H. Taira, and P. Lengyel.** 1978. Interferon, double-stranded RNA, and protein phosphorylation. J. Biol. Chem. **253**:5915–5921.
59. **Sonenberg, N.** 1990. Measures and countermeasures in the modulation of initiation factor activities by viruses. New Biol. **2**:402–409.
60. **Svensson, C., and G. Akusjärvi.** 1985. Adenovirus VA RNA_I mediates a translational stimulation which is not restricted to the viral mRNAs. EMBO J. **4**:957–964.
61. **Tiwari, R. K., J. Kusari, R. Kumar, and G. C. Sen.** 1988. Gene induction by interferons and double-stranded RNA: selective inhibition by 2-aminopurine. Mol. Cell. Biol. **8**:4289–4294.
62. **Torrence, P. F., M. I. Johnston, D. A. Epstein, H. Jacobsen, and R. M. Friedman.** 1981. Activation of human and mouse 2-5A synthetases and mouse protein P_1 kinase by nucleic acids. FEBS Lett. **130**:291–296.
63. **Zilberstein, A., A. Kimchi, A. Schmidt, and M. Revel.** 1978. Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. Proc. Natl. Acad. Sci. USA **75**:4734–4738.
64. **Zinn, K., A. Keller, L.-A. Whitemore, and T. Maniatis.** 1988. 2-Aminopurine selectively inhibits the induction of β-interferon, *c-fos*, and *c-myc* gene expression. Science **240**:210–213.